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Analysis of Heavy-Chain Antibody Responses and Resistance to *Parelaphostrongylus tenuis* in Experimentally Infected Alpacas

S. R. Purdy,[a] L. F. Gagliardo,[b] S. Lefman,[a] P. J. S. Hamel,[a] S. Ku,[a] T. Mainini,[a] G. Hoyt,[a] K. Justus,[b] L. P. Daley-Bauer,[b]* M. S. Duffy,[c] and J. A. Appleton[b]

Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Hadley, Massachusetts, USA[a]; Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA[b]; and Department of Biology, University of New Brunswick, Fredericton, New Brunswick, Canada[c]

The parasitic nematode *Parelaphostrongylus tenuis* is an important cause of neurologic disease of camelids in central and eastern North America. The aim of this study was to determine whether alpacas develop resistance to disease caused by *P. tenuis* in response to a previous infection or a combination of controlled infection and immunization. Alpacas were immunized with a homogenate of third-stage larvae (L3) and simultaneously implanted subcutaneously with diffusion chambers containing 20 live L3. Sham-treated animals received adjuvant alone and empty chambers. The protocol was not effective in inducing resistance to oral challenge with 10 L3, and disease developed between 60 and 71 days following infection. Immediately following the onset of neurologic disease, affected animals were treated with a regimen of anthelmintic and anti-inflammatory drugs, and all recovered. One year later, a subset of alpacas from this experiment was challenged with 20 L3 and the results showed that prior infection induced resistance to disease. Primary and secondary infections induced production of conventional and heavy-chain IgGs that reacted with soluble antigens in L3 homogenates but did not consistently recognize a recombinant form of a parasite-derived aspartyl protease inhibitor. Thus, the latter antigen may not be a good candidate for serology-based diagnostic tests. Antibody responses to parasite antigens occurred in the absence of overt disease, demonstrating that *P. tenuis* infection can be subclinical in a host that has been considered to be highly susceptible to disease. The potential for immunoprophylaxis to be effective in preventing disease caused by *P. tenuis* was supported by evidence of resistance to reinfection.

Parelaphostrongylus tenuis is a parasitic nematode that is endemic in central and eastern North America. The definitive host is the white-tailed deer, *Odocoileus virginianus*, and terrestrial gastropods serve as intermediate hosts. A variety of grazing animals that are sympatric with white-tailed deer can be exposed to *P. tenuis* by consuming infected gastropods or vegetation contaminated with the third-stage larvae (L3) that emerge from them (13). Infections are asymptomatic in white-tailed deer; however, in other susceptible species, the parasite migrates aberrantly and can cause severe neurologic disease (reviewed in reference 26). It is well established that other cervids, including elk and moose, as well as camelids, sheep, and goats are susceptible to infection (4). Disease caused by *P. tenuis* has been described in horses (36, 41), cattle (T. J. Divers, Cornell University, personal communication), and bison (45), documenting that the nematode has the capacity to infect a broad range of hosts.

Parasitic worms are an important cause of morbidity in domestic animals. Control measures have relied heavily on anthelmintic drugs, and it has become evident in recent years that drug resistance has emerged in populations of parasitic worms of small ruminants around the world (reviewed in reference 43). Because disease caused by *P. tenuis* in camelids is often permanently debilitating or results in euthanasia, routine anthelmintic treatment has been used as a preventative. Monthly treatment with ivermectin during the calendar months corresponding to the greatest risk for exposure is widely recommended (26). Drug resistance in *P. tenuis* does not develop as a result of this practice, as the parasite does not reproduce in camelids; rather, the hazard is associated with other worms that parasitize camelids. Recent data from the state of Georgia indicate that gastrointestinal parasites of camelids are emerging that are resistant to two of three major classes of anthelmintic drugs (18). Efforts to develop new antihelmintic drugs have been limited in recent years, affording little promise for new chemical preventatives (17). The impact of environmental contamination with anthelmintics raises additional concern. These issues prompt consideration of other prophylactic approaches, including vaccination.

A goal of this investigation was to determine whether immunization or experimental infection would induce resistance to disease in alpacas. There are published reports of experimental and natural infections of llamas with *P. tenuis* (7, 16, 22, 37), but reports concerning alpacas are limited to one case description (23). Studies in llamas, as well as other susceptible species, were largely focused on evaluation of susceptibility to infection and description of clinical parameters of disease. Investigations of the immune response to infection in any species have emphasized development of serodiagnostic tools (14, 27, 30–34). The question of whether susceptible species develop resistance to infection has not been addressed experimentally.

A second goal of this study was to investigate the antibody (Ab) response to larval antigens of *P. tenuis* during the course of infection. Although camelids produce conventional, tetrameric IgG,

Received 1 April 2012 Returned for modification 18 April 2012
Accepted 7 May 2012
Published ahead of print 16 May 2012
Address correspondence to J. A. Appleton, jaa2@cornell.edu.
* Present address: L. P. Daley-Bauer, Department of Microbiology & Immunology, Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia, USA.

doi:10.1128/CVI.00178-12

they also produce immunoglobulins that are comprised solely of heavy chains (HC) (19). Previously, we have shown that production of these so-called HC IgGs is differentially regulated during viral infection and that the isotypes differ in their capacities for virus neutralization (10). In the present investigation, we quantified HC IgGs in the response to a nematode infection. The data provide a foundation for further investigation of the role of HC IgG in protection. Conventional antibodies are known to be protective in nematode infections (5, 6, 20). The structure of the variable domains of HC IgGs is such that they are effective inhibitors of enzymes (8, 12, 15, 42), and nematode proteases are numerous and prominent at the host-parasite interface (13, 35, 38–40). Thus, HC IgGs have unique potential to contribute to protective immunity against nematodes.

Our findings indicate that alpacas are susceptible to low doses of L3 *P. tenuis*, that infection can be subclinical and yet induce strong and diverse IgG responses, and that prior infection induces resistance to disease. The results support the merit of further investigation of active immunization as a preventative against disease caused by *P. tenuis*.

MATERIALS AND METHODS

Alpacas. Neutered male, adult Huacaya alpacas (3 to 5 years of age) weighing approximately 150 to 180 pounds were transported from central Washington state, where *P. tenuis* is not endemic, to South Deerfield, MA, where the study was conducted. In Washington, these animals had been maintained on pasture as part of a large herd and were subjected to routine deworming and vaccination. Upon arrival in Massachusetts, a physical examination was performed and each alpaca was vaccinated against rabies and dewormed with fenbendazole paste (Panacur; Intervet-Schering-Plough, Millsboro, DE). Alpacas were fed grass hay and livestock grain *ad libitum* and were housed on sawdust in a large barn that was open to a paddock. Because Massachusetts is a state in which *P. tenuis* infection is endemic, the barn and paddock were maintained free of vegetation in order to reduce the risk of exposure to infected gastropods. Animals were acclimated for 2 weeks prior to initiation of the first experiment. Animal care and use was approved by the IACUC at the University of Massachusetts at Amherst.

Antibodies. Mouse monoclonal antibodies (MAbs) specific for conventional and heavy-chain isotypes of llama IgG have been previously described (9, 11) and were detected with peroxidase-conjugated goat anti-mouse antibodies (ICN/Cappel, Aurora, OH). The monoclonal antibodies detect conventional IgG1 (clone 27E10), HC IgG2 (clone 19D8), and HC IgG3 (clone 1D1, which cross-reacts with two species of IgG3 [11]).

Parasite and antigens. First-stage *P. tenuis* larvae were obtained from feces of two experimentally infected white-tailed deer, as previously described (14). Third-stage *P. tenuis* larvae were recovered from laboratory-reared terrestrial gastropods (*Triodopsis* sp.) that had been infected at least 90 days prior with L1. Larvae were recovered from snail tissue by digestion in 0.6% pepsin–90 mM HCl (3). For infection of alpacas, L3 were washed in Dulbecco's phosphate-buffered saline (DPBS) and 10 or 20 larvae were suspended in 2.5 ml of a solution of 0.6% nutrient broth and 2% gelatin.

For *in vitro* culture, L3 were washed with sterile 0.85% NaCl and suspended in 1 ml of DMEM plus streptomycin/penicillin, with or without 10% fetal bovine serum, and cultured at 37°C with 8% CO_2 in 48-well plates. Medium was changed every third day, and larvae were observed daily with a dissecting microscope over a 30-day period.

L3 antigens for immunization were prepared by snap-freezing and thawing L3 three times, followed by homogenization in DPBS using a ground glass homogenizer. The final suspension contained both soluble and insoluble components of L3. Each alpaca was injected with lysate derived from 500 L3 (first injection), 250 L3 (second and third injections), or 300 L3 (fourth injection), estimated to incorporate 140, 70, 70, and 84 μg of soluble protein, respectively. Lysates were mixed with an equal volume of adjuvant according to the manufacturer's instructions (Imject Alum; Pierce, Rockford, IL).

Soluble L3 antigens used for enzyme-linked immunosorbent assays (ELISA) were prepared by snap-freezing L3 in liquid nitrogen, thawing at 37°C in the presence of complete protease inhibitor cocktail (Roche, Indianapolis, IN) (used at twice the recommended concentration), sonicating on ice (Branson Sonifier 450) until worms were thoroughly fragmented, and then centrifuging at 5,000 × *g* to remove particulates. Protein concentration was determined using a Bradford protein assay (Bio-Rad, Hercules, CA). Antigens were stored at −20°C. Preparation of a recombinant form of an aspartyl protease inhibitor from *P. tenuis* (rPt-API) has been described elsewhere (14).

Immunization and challenge of alpacas. In the first experiment (Fig. 1A), groups of 5 alpacas were injected subcutaneously in the neck with L3 homogenate ("immunized") or PBS ("sham") mixed with an equal volume of adjuvant. Injections were given on days 1, 20, 56, and 88 of the experiment (2-ml total volume on days 1, 20, and 88; 5-ml total volume on day 56). In addition, on days 1, 20, and 56, a diffusion chamber constructed with a 5.0-μm-pore-size Durapore membrane (obtained from David Abraham, Thomas Jefferson University [2]), containing either saline solution or 20 live L3 in saline solution, was surgically implanted under the skin of the dorsolateral thorax of each animal. Animals in the sham treatment group received chambers containing saline solution only. Animals in the immunized group received chambers containing L3 in saline solution. Chambers were replaced on days 20 and 56, and the last chamber was removed on day 77. Five animals served as sentinels for uncontrolled (natural) exposure to the parasite and were neither injected nor implanted with chambers. On day 108, animals in the immunized and sham treatment groups received 10 *P. tenuis* L3 (suspended in 2.5 ml of 2% gelatin–0.6% nutrient broth) delivered orally with a syringe. Animals were observed for signs of disease for 5 months.

One year after oral infection, three animals from the immunized group, three animals from the sham group, and four sentinel animals from the first experiment were infected orally with 20 L3. Among the alpacas in the immunized and sham groups, three had developed disease in the first experiment and three had not. Two additional, previously uninfected animals served as sentinels and were not infected. Alpacas were observed for signs of disease for 4 months.

Blood and cerebral spinal fluid collection and analysis. Blood samples were obtained by jugular venipuncture from alpacas every 14 days throughout the course of each experiment. In the immunization study, total and differential white blood cell counts, as well as total protein and packed cell volume measurements, were performed. Serum for antibody measurement was separated by centrifugation and stored at −20°C. In the second experiment, a sample of cerebrospinal fluid (CSF) was obtained by lumbar puncture at the time of onset of neurologic disease. Samples were collected into EDTA tubes and refrigerated until evaluation for cellularity.

Assessment of disease. Animals in all groups were observed daily for evidence of disease. Testing of affected animals included manually restraining the alpaca by the neck and applying firm pressure over the hips to test strength; pushing the animal from the side of the pelvis laterally to the left, and then to the right, while watching for a fluid, hopping motion; spinning the alpaca quickly in a circle in both directions; and observing the animal walking and running untouched. Abnormal signs included evidence of weakness in the hindquarters when downward pressure was applied, for example, sinking to the ground; dragging or crossing of limbs when pushed in a circle; lameness or tripping when walking; favoring certain limbs; leaning on the experimenter when pushed; difficulty rising from a prone position; and a basewide positioning of the legs when standing.

Treatment of affected animals. The following treatment was initiated on the day that clinical signs were first observed: fenbendazole paste (Panacur; Intervet-Schering-Plough, Millsboro, DE) (10 mg/kg of body weight by oral administration) and flunixin meglumine (Flunixamine; Fort Dodge Animal Health, Overland Park, KS) (1.5 ml of 50 mg/ml by

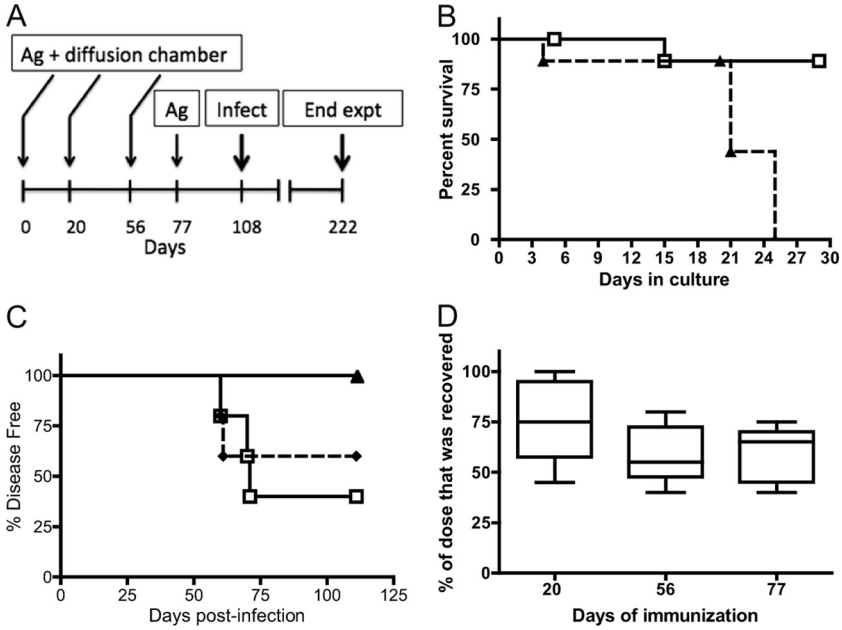

FIG 1 (A) Experimental design for immunization and challenge of alpacas. Alpacas were immunized with antigen mixed with adjuvant or with adjuvant alone on days 0, 20, 56, and 77. Diffusion chambers with or without L3 were implanted on day 0, 20, or 56 and then removed on the next immunization date. Alpacas were infected on day 108, and animals were observed through day 222. (B) Survival of *P. tenuis* larvae cultured in the presence (squares) and absence (triangles) of 10% fetal bovine serum. $n = 9$ larvae per group. (C) Disease development in immunized (squares) and sham-treated (diamonds) alpacas infected with 10 *P. tenuis* L3. Sentinel alpacas did not develop disease (triangles). $n = 5$ animals per group. (D) Recovery of intact L3 (live and dead) from diffusion chambers during the course of immunization. Horizontal bars indicate the median, and error bars indicate the standard deviation from the mean. $n = 5$ chambers per time point.

intramuscular injection) once daily for 5 days and 1% ivermectin (Noromectin; Norbrook Laboratories, Newry, United Kingdom) (8 ml by subcutaneous injection) and dimethyl sulfoxide (DMSO) solution (75 ml of DMSO plus 225 ml of water by oral administration) on alternate days for three treatments. Both blood collection and observation were continued for the duration of the study for all treated animals. At the conclusion of the first experiment, all alpacas were treated with 1% ivermectin (1.5 ml; subcutaneous) and then turned out to pasture. Ivermectin was injected monthly at the same dose until alpacas were brought back into the enclosure for the second experiment 6 months later.

Detection of alpaca IgGs specific for *P. tenuis* antigens. ELISA was performed as described previously (9, 11), with modifications. Polyvinyl microtiter plates were coated at 4°C overnight with r*Pt*-API (5 μg/ml) or soluble L3 antigens in 10% DPBS. Subsequent incubations were conducted at room temperature, and wells were washed with DPBS containing 0.05% Tween 20. Wells were blocked with 50 μl of DPBS containing 2% skim milk–0.05% Tween 20 and then incubated with alpaca serum diluted 1:100 in blocking solution. After washing, wells were incubated with 27E10 (IgG1), 19D8 (IgG2), or 1D1 (IgG3) MAb (5 μg/ml) diluted in blocking solution. Bound MAbs were detected with peroxidase-conjugated goat anti-mouse antibodies (5 μg/ml) diluted in blocking solution containing 10% normal goat serum. The assay was developed with 3,3,5,5′-tetramethyl-benzidine (KPL, Gaithersburg, MD), and reactions were terminated with 1 M H_3PO_4. Optical densities (OD) were measured at 450 nm.

Statistical analysis. Susceptibilities to disease were compared among treatment groups by applying the log rank test to survival curves. Recovery and viability of larvae in chambers at each time point were analyzed using analysis of variance (ANOVA) and Tukey's test. Antibody concentrations were compared using paired or unpaired *t* tests where indicated. All analyses were performed with Prism software (GraphPad Software, La Jolla, CA).

RESULTS

Survival of *P. tenuis* L3 *in vitro*. We conducted an *in vitro* experiment in order to estimate how long L3 would survive in diffusion chambers. Figure 1B shows that 100% of L3 incubated at 37°C in cell culture medium supplemented with fetal bovine serum remained viable (as evidenced by motility, body integrity, and translucence) for 15 days and that 89% survived for 30 days. In serum-free medium, all larvae were dead after 25 days in culture. Based on these results, we designed the immunization protocol such that diffusion chambers containing larvae would remain implanted for periods of 20 to 36 days (Fig. 1A).

Effect of immunization on experimental infection with *P. tenuis*. Between 60 and 71 days postinfection (dpi) with 10 *P. tenuis* L3, five alpacas developed signs of disease: three in the immunized group and two in the sham treatment group (Fig. 1C). Affected animals displayed a variety of signs, including moderate hind limb weakness, mild lameness, dragging a hind leg, ataxia, and difficulty circling. The nature or severity of the signs did not correlate with immunization history. Sentinel alpacas did not develop disease, confirming that the animals were not naturally exposed to the parasite during the 8 months of experimentation.

Immune responses to immunization and experimental infection with *P. tenuis*. There were no significant changes in total blood leukocyte numbers, differential leukocyte counts, total protein concentrations, or packed cell volumes in any of the animals during the course of infection (data not shown). Similar results have been reported for experimentally infected llamas during the period between exposure and onset of disease (22, 37).

After being removed from the subcutaneous implantation site, chambers were filled with a fibrous gel and most larvae were em-

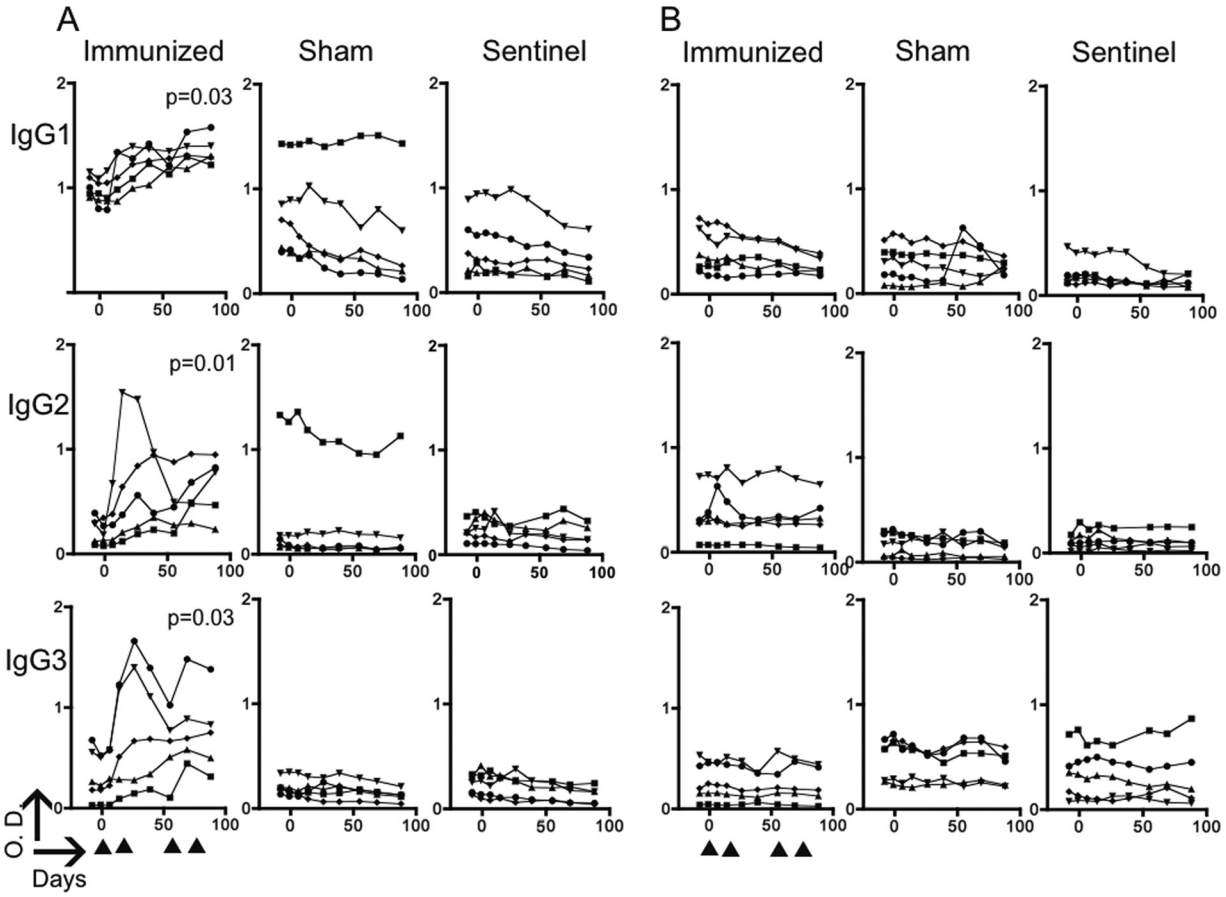

FIG 2 Detection of conventional and HC IgGs specific for (A) L3 soluble antigens or (B) r*Pt*-API in sera of immunized, sham-treated, and sentinel alpacas. Sera were collected at the intervals indicated prior to immunization (days −8 to 0) and during immunization (days 0 to 88). Antibodies were detected in sera by ELISA using isotype-specific reagents. Values reported are optical densities (OD) for serum samples tested at a dilution of 1:100. Symbols within each group are assigned to the same animals as those indicated in Fig. 3 and 5. Arrowheads indicate the dates of immunization. *P* values indicate significant differences between group means on day −1 versus day 88 as determined in an upaired *t* test.

bedded in this material. The viability of the larvae recovered was higher in chambers removed after 20 days than in those removed after 56 and 77 days (96% versus 80% and 80%, respectively). The number of larvae recovered from chambers after 20 days of immunization was greater than at either of the two subsequent time points, although the difference was not statistically significant (Fig. 1D). Overall, the immune response to L3 during the immunization protocol induced a maximum elimination/killing rate of 50% of the larvae in the chambers.

Immunization induced IgG of both conventional and HC isotypes, which reacted with soluble L3 antigens in crude lysates (Fig. 2A). Animals in all groups had high levels of cross-reactive IgG1 prior to the start of the experiment (10 of 15 animals with an OD value of > 0.5), while preexisting, cross-reactive HC IgGs were detected less frequently (only 4 of 15 animals with an OD value of >0.5 for either IgG2 or IgG3). This result is consistent with our previously published findings in naturally infected llamas (9). Immunization induced antigen-specific IgG1, IgG2, and IgG3 at levels that increased significantly between 1 and 88 dpi (*P* values of 0.03, 0.01, and 0.03, respectively, determined in unpaired *t* tests). Preexisting antibody levels generally declined over time in sera of alpacas in the sham treatment and sentinel groups, which is compatible with the conclusion that they had been induced by a nematode infection that was cleared by anthelmintic treatment prior to initiating the study.

In addition to soluble L3 antigens, sera were tested for antibodies specific for r*Pt*-API (Fig. 2B). Preexisting, cross-reactive antibodies were present in several alpacas across all three groups. With the exception of one weak IgG2 response, the immunization protocol did not induce antibodies that reacted with r*Pt*-API.

Among sham-treated alpacas, L3 infection on day 108 of the protocol induced significant increases in levels of specific antibodies of all isotypes, with the most dramatic changes evident in IgG1 and IgG3 (average increases of 0.875 and 0.582 OD units, respectively). Responses peaked after 36 to 50 days and showed a steady decline in most animals for the remainder of the experiment (Fig. 3A). In immunized alpacas, specific serum IgG2 and IgG3 antibodies increased (average increases of 0.425 and 0.387 OD units, respectively), while the IgG1 response was less dramatic (average increase of 0.164 OD units). Elevated levels of IgG1 and IgG3 were sustained over a prolonged period in the immunized group, and some of these animals mounted a second IgG3 response (3 of 5 alpacas) and a second IgG2 response (2 of 5 alpacas) between 80 and 100 dpi. This second response was not observed in the sham treatment group. In one alpaca, disease developed and treatment was initiated at 71 dpi, so this late boost in serum antibodies may

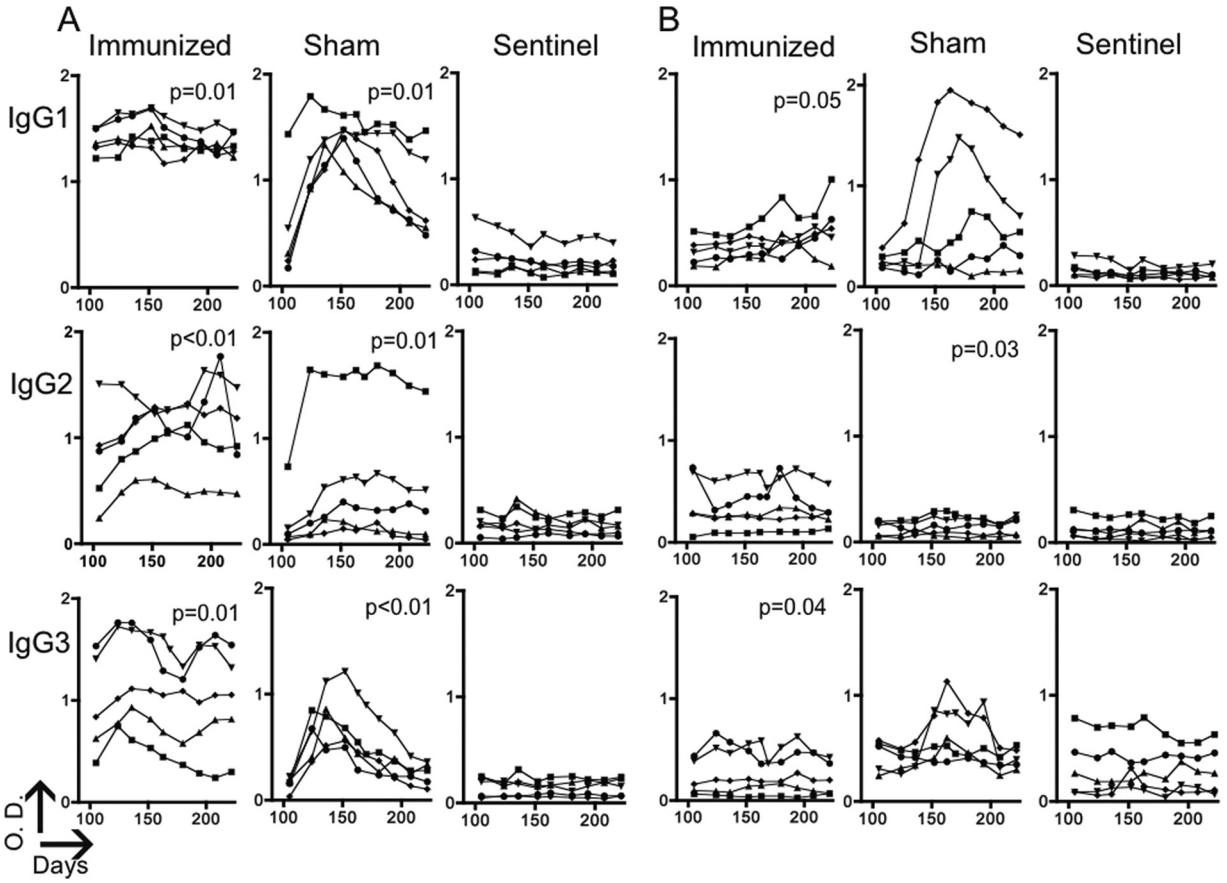

FIG 3 Detection of conventional and HC IgGs specific for (A) L3 soluble antigens or (B) r*Pt*-API in sera of immunized, sham-treated, and sentinel alpacas following infection of immunized and sham-treated groups with 10 L3 on day 105. Antibodies were detected as described in Fig. 2. Symbols within each group are assigned to the same animals as those indicated in Fig. 5. *P* values indicate significant differences between OD values on day 88 versus day 152 postinfection as determined in a paired *t* test.

have correlated with parasite death and release of antigens; however, the other alpacas did not show signs of disease and were not treated with ivermectin until the end of the experiment. The biphasic change occurred only in the HC isotypes and only in immunized animals. The observation suggests a change in activity for *P. tenuis* in immunized animals that prompted either a primary antibody response to a novel antigen or a secondary response to antigens that had been introduced during immunization.

Oral infection with L3 induced highly variable antibody responses to r*Pt*-API. Although very strong IgG1 responses were induced in some alpacas in the sham treatment group, overall the change was not statistically significant. The results raise concerns about sensitivity as well as specificity of r*Pt*-API as a diagnostic antigen for serologic detection of infection in alpacas.

The strength of the antibody response to immunization did not predict disease development; immunized alpacas that developed disease were neither the strongest nor the weakest responders. In the sham treatment group, all five animals responded to infection with strong IgG1 and IgG3 responses to crude L3 antigens, but only two developed disease, supporting the conclusion that alpacas can experience subclinical infections with *P. tenuis*, even during a primary infection. When immunized and sham-treated alpacas were infected, levels of antigen-reactive serum antibodies increased at least 2 weeks prior to the onset of disease, suggesting that a serologic test may be useful in diagnosis.

Outcome of secondary infection with *P. tenuis*. One year after the first infection with 10 L3, a subset of immunized, sham-treated, and sentinel alpacas were challenged orally with 20 L3 to test their resistance to infection. Animals from the immunized and sham treatment groups had mounted antibody responses during the first experiment, indicating that they had experienced active infections. Of these previously exposed animals, only one of six developed disease following secondary infection with 20 L3. This animal was in the immunized group and had shown signs of disease in the first experiment; two other animals that were diseased in the first experiment did not develop disease in the challenge experiment. In contrast, four of four of the alpacas that had not been infected previously developed disease. The difference between the previously infected group and the previously uninfected group was statistically significant by the log rank test ($P = 0.026$; Fig. 4). Among the five affected animals, four developed disease between 43 and 55 days postinfection; one alpaca developed disease after 86 days. Two sentinel alpacas did not develop disease, confirming that the parasite did not contaminate the environment during the 4 months of experimentation.

Immune responses to secondary infection with *P. tenuis*. Following infection with 20 L3, previously infected alpacas

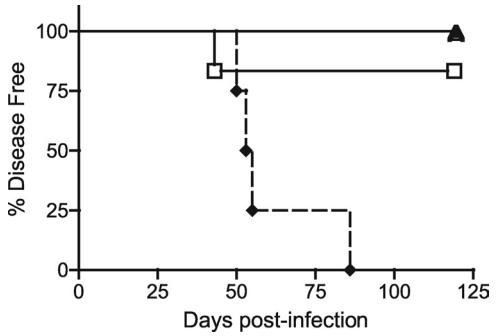

FIG 4 Disease development in previously infected alpacas following reinfection with 20 L3. Previously infected (squares) alpacas included sham-treated and immunized animals from the previous experiment ($n = 6$). Previously uninfected alpacas (diamonds; $n = 4$) served as positive controls, and sentinel alpacas (triangle; $n = 2$) served as infection controls. Curves for previously infected versus previously uninfected alpacas were significantly different ($P = 0.026$).

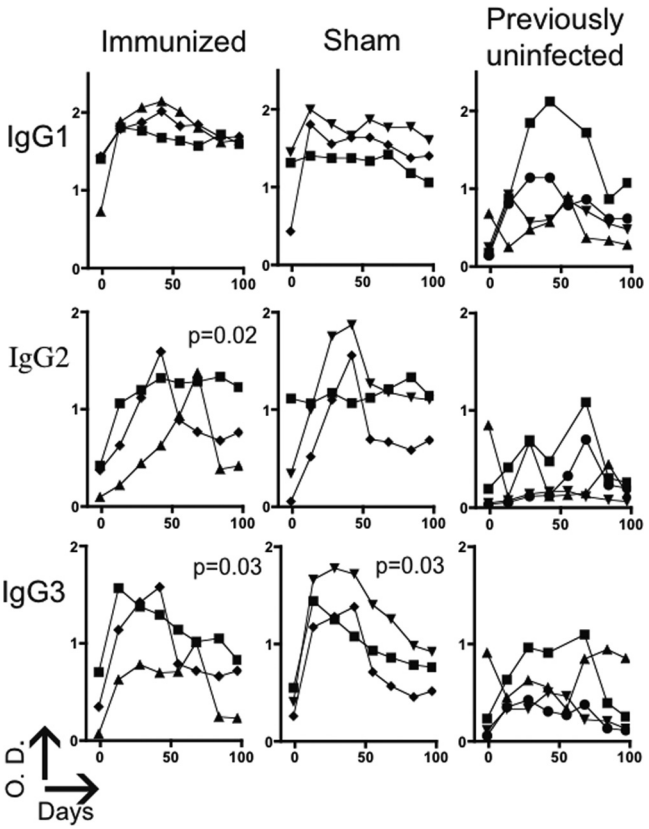

FIG 5 Detection of conventional and HC IgGs specific for L3 soluble antigens in sera of previously infected (immunized and sham immunized) and previously uninfected alpacas following infection with 20 *P. tenuis* L3. Infection occurred on day 0, and sera were collected at the intervals indicated following infection through day 97. Antibodies were detected as described for Fig. 2. Symbols within each group are assigned to the same animals as those indicated in Fig. 2 and 3. *P* values indicate significant differences between OD values on day −1 versus day 42 postinfection as determined in a paired *t* test.

mounted stronger and more rapid antibody responses than did previously uninfected animals (Fig. 5). One animal (sham treatment group in the first experiment) failed to produce IgG1 and IgG2 in response to challenge infection but did mount a strong IgG3 response, while the other animals that had been infected previously produced antibodies of each isotype. Responses in previously uninfected animals did not show any obvious correlation with onset of disease; specifically, the animal that first showed signs of disease 86 days postinfection (circle symbol in Fig. 5) did not mount an antibody response that was distinguishable from those of the other three alpacas that showed signs of disease between 50 and 55 days postinfection.

Cerebrospinal fluid analysis. Although we did not compare CSF eosinophil numbers among the different groups of animals, CSF samples collected at the time of onset of neurologic disease in affected alpacas (following oral infection with 20 L3) showed eosinophil values ranging from 70% to 90% (normal range is 0% to rare), providing further confirmation of active nematode infections in these diseased animals.

Response to treatment. The therapeutic regimen described was effective in halting the progression of disease in *P. tenuis*-infected alpacas. All animals that were treated during the first experiment recovered fully within 1 week. Similar results were obtained in the challenge experiment.

DISCUSSION

The immunization protocol in our study was designed on the presumption that *P. tenuis* L3 secrete or display immunogenic molecules that are necessary for establishing infection. Larvae implanted in diffusion chambers served two purposes. First, they would release products important in host invasion, thereby boosting desirable antibody specificities in the immune response induced by crude larval homogenates. Second, larvae in chambers served as sentinels for immune responses directed against the parasite. Used extensively in studies of immunity to a variety of filarial worms (1, 24, 25, 44), as well as *Strongyloides stercoralis* (2), diffusion chambers are constructed with membranes of different pore sizes in order to allow access of leukocytes, as was the case in the present study, or to exclude them. In this way, the roles of soluble and cellular mediators in parasite clearance have been discerned *in vivo* (21, 28, 29). The results of our experiment show that the immunization protocol, which combined larvae in diffusion chambers with administration of adjuvanted crude larval homogenates, was effective in inducing significant antibody responses; however, the impact on larvae in chambers was modest and there was no protection evident when alpacas were challenged 1 month after the final immunization. Implementation of the treatment protocol immediately upon presentation of neurologic signs precluded evaluation of the impact of immunization on disease progression.

In contrast to these findings, oral infection with 10 L3 induced resistance to disease caused by reinfection 1 year later, an interval that corresponds to the annual, seasonal nature of exposure to *P. tenuis*. This result suggests that larval migration and associated injury during natural infection may induce innate and cellular responses that are distinct from those induced by larvae confined to a diffusion chamber in an unnatural anatomic site. Alternatively, L3 may be induced to express different genes, or to display or release different products, during natural infection. The crude larval extract used in ELISA contains a large number of potential antigens. Comparison of the specificities of antibody responses induced by natural infection versus failed immunization identifies antigens targeted in protective immunity. Samples collected in this study would be useful for such an analysis.

A variety of treatment protocols has been described in the clinical literature, but there have been no controlled studies of treatment efficacy (reviewed in reference 26). The treatment regimen described here was effective when applied to affected animals in both experiments. It is likely that the favorable treatment outcomes were facilitated by early diagnosis associated with daily observation and immediate initiation of the protocol, as has been suggested by others (26). In contrast to the controlled conditions of this experimental study, observation is infrequent when animals are grazing as part of a breeding or fiber herd and this treatment protocol has been less effective when applied in veterinary farm practice (S. R. Purdy, unpublished observations). Effective treatment of experimentally infected alpacas made it possible to conduct the second challenge trial and should be a useful tool in future studies.

The presence of IgG that reacted with *Pt*-API in sera of alpacas prior to infection with *P. tenuis* was likely induced by infection with some other nematode, consistent with the conserved nature of this protein among parasitic worms. Thus, *Pt*-API does not appear to have value as an antigen for serodiagnosis in alpacas. Similarly, antibodies that cross-reacted with unidentified antigens in the crude extract of L3 were present in uninfected alpacas. Infected alpacas did produce serum antibodies specific for antigens in crude larval extracts at least 2 weeks prior to the onset of disease, suggesting that a serologic test with a particular L3 antigen may be useful in diagnosis.

Production of IgG1 and IgG3 specific for r*Pt*-API in some alpacas following oral infection with *P. tenuis* was similar to that described previously in experimentally infected red deer (14). In that study, levels of r*Pt*-API-specific serum IgG peaked and then declined between 70 and 100 days postinfection but increased again between 100 and 200 days postinfection in animals that developed patent infections. Alpacas do not develop patent infections, and in our study, infected alpacas that did produce antibodies to r*Pt*-API did not mount a second late response to this antigen. Nevertheless, some animals in the immunized group did mount a second HC IgG response to antigens in extracts of L3 that peaked at approximately 100 dpi, suggesting some change in parasite activity or antigen release at this time point.

We have reported previously that HC and conventional IgGs are induced by immunization with a vaccine that incorporates West Nile virus and alum (10). Similar results were obtained in this study with crude *P. tenuis* homogenates prepared with alum. In contrast, natural or experimental infections of alpacas with West Nile virus induced conventional IgG1 and HC IgG3, with poor induction of HC IgG2, while *P. tenuis* infection induced both HC IgG2 and IgG3. The results suggest that the two HC isotypes are regulated by distinct mechanisms. In general, secondary infection with *P. tenuis* induced strong responses of all IgGs, indicating that memory B cells develop for each isotype. This finding is consistent with the location of HC and conventional IgG^+ B cells in germinal centers of lymphoid tissues (11). Previously, we reported that there are functional distinctions among isotypes. Although all IgGs are transferred in colostrum, maternal HC isotypes have shorter half-lives in neonatal circulation (11). All IgGs bind to the surfaces of macrophages and enhance the infectivity of West Nile virus for cultured macrophages (10); however, IgG2 performs poorly in virus neutralization whereas IgG3 neutralizes virus as effectively as conventional IgG1 (10). Functional attributes of HC IgGs that may be relevant to nematode infection have not been described, and additional studies to address this question are warranted.

In summary, we have shown that alpacas mount sustained and diverse antibody responses to *P. tenuis* in the context of both clinical and subclinical infections. Furthermore, animals experiencing subclinical infection or, alternatively, disease that is controlled by immediate treatment show resistance to secondary infection. These findings provide motivation for further investigation of immune prophylaxis as a tool for preventing disease caused by *P. tenuis*. A vaccine would reduce dependency upon anthelmintic approaches to *P. tenuis* prevention that coincidently promote drug resistance in gastrointestinal nematodes of camelids.

ACKNOWLEDGMENTS

We thank David Abraham for helpful discussions and Scott Soprano for assistance with snail husbandry.

This work was supported by gifts from Ian and Jennifer Lutz of Cas-Cad-Nac Farm Alpacas (Perkinsville, Vermont), the Pennsylvania Alpaca Breeders and Owners Association, the Mid-Atlantic Alpaca Owners and Breeders Association, Alpacas of America (Tinino, Washington), and other anonymous donors.

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